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Aeromonas salmonicida: Causal Agent of Furunculosis in Salmonids Identification and Therapeutic Experimentation In Vitro

M. Truquet(a) and G. Michel(b)

Introduction

Aeromonas salmonicida, the causal agent of furunculosis in salmonids, is responsible for recurrent enzootic disease in intensive fish breeding stations. Some investigators (1,2) have shown that the disease also occurs in other fish families. As a consequence, it is of considerable interest both scientifically and economically.

Clinically, the disease occurs in the form of furuncles on the body of the fish. Since symptoms are not always so clearcut, it is essential to identify the pathogen by microbiological methods. In this study, we designed a series of precise tests, based on the work of various authors (1,3,4,5,6) in order to identify the organism. The in vitro effectiveness of new and traditional treatments will be discussed on the strength of our experimental findings.

Materials and Methods

A. Bacterial Strains

Our sampling was intentionally diversified and included the following:

- two strains from a Czechoslovak collection (c)
- twenty strains isolated at the INRA Ichthyopathology Laboratory (d)

(a) Doctor of pharmacy; diploma 1982; Toulouse Faculty of Pharmaceutical Sciences

(b) Professor Georges Michel, Director, Industrial Microbiology and Virology Laboratory

(c) Czechoslovak Collection of Microorganisms -J.E. Purkyne University, 662.43 Brno, Czechoslovakia

- three strains from a roach and a trout (GF 28, TR 24 and TR TR 24 Ed)

The latter two strains were isolated either directly (TR 24) or following incubation of preliminary cultures (TR 24 Ed) on tryptocasein soy broth (e) at 22°C.

Microbiological samples were taken from frozen fish on September 21, 1982, i.e., two to six months after the specimens had been caught.

All of the strains were grown on tryptocasein soya agar at room temperature (22°C). Subcultures were made every week on tryoptocasein soya broth.

B. Identification Methods

Identification was based on morphological, physiological and biochemical characteristics (1,4,5,6). The first tests involved determining bacterial shape and motility. Physiological tests involved an analysis of growth and pigment production. These two tests were carried out simultaneously on each strain at two different temperatures , i.e., 22°C and 37°C, one of which proved lethal for the bacterium (f). In addition to the PBG agar test (3), we selected 15 different reactions among the most representative biochemical tests for Aeromonas salmonicida.

(d) National Institute for Agronomic Research - Ichthyopathology Laboratory - Route de Thiverval - 78850 Thiverval Grignon

(e) Bio Mérieux

(f) Pseudomonas is capable of producing pigment at 22° as well as at 37°C (Hamilton-Miller, 1975). This reaction consequently constitutes a valuable differential test.

C. Antibacterial Agents. Selection and Experimentation

The following antibiotics were selected on the basis of effectiveness and cost price and subsequently tested: ampicillin, chloramphenicol, co-trimoxazole, nitrofurantoin and flumequine. The antisentics and disinfectants selected included AMMO 4 B (dimethyl lauryl benzylammonium bromide), cetrimide, chlorhexidine, povidone-iodine and merthiolate.

A series of dilutions was prepared for each compound in order to determine product activity expressed in terms of the minimum inhibitory concentration (g). The latter was established from the mean recommended therapeutic dose (1, 10, 11). Antibiotics are subject to complex absorption and distribution phenomena in various parts of the organism. Since it was beyond the scope of this bacteriological study to elucidate these phenomena, we were obliged to assume a priori that the antibiotics distributed in the food were completely absorbed in the digestive tract, that they were uniformly distributed throughout the organism and, lastly, that individual fish size was, by and large, commensurate with fish weight. On the basis of these assumotions, the prescribed dose of antibiotics had to be at least equal to the minimum inhibitory concentration to be of therapeutic value.

(g) The minimum inhibitory concentration of each substance corresponds to the weakest dose capable of preventing growth of the initial inoculum over a 48-hour period.

Petri dishes containing diluted antimicrobial agents in trypto-casein soya agar were prepared extemporaneously. Concentrations of 48-hour bacterial cultures were made uniform with the aid of a photometer before the cultures were deposited on the medium. Concentrations of 10^5 bacteria per microlitre were obtained by correctly diluting the culture in trypticase soya broth until they produced an optical density reading of 85 on the photometer. Once the 23 strains had been standardized, they were deposited on the agar surface by means of a Multipoint inoculator.

Results

Our findings are shown in Tables 1 and 2.

A. Identification - Table 1

A comparison of identification reactions yielded the following results:

- Strains 05.74 and GF 28 were not Aeromonas salmonicida organisms and were consequently discarded from the study.

- All the other strains reacted unequivocally to the range of tests selected. Only small variations were recorded.

B. Organism Susceptibility - Table 2.

Minimum concentrations of inhibition were expressed in mg/l in the case of all the antibiotics and antiseptics, except AMMO 4 B (ml/ml) and Betadine (g/l).

The various findings could be summarized by the dose capable of effectively producing 100% inhibition (effective dose 100). On this basis, the antibiotics were classified in decreasing order of effectiveness with flumequine heading the list, followed by ampicillin, nitrofurantoin, Bactrim and lastly by chloramphenicol (0.4 mg/l

TESTS / SOURCES STRAINS	Form	Motility	Growth at 22°	Growth at 37°	Pigment at 22°	Pigment at 37°	Gram	Cyt. oxidase	LDC	ADH	ODC	Indole	H_2S production	Red. Nitrate	Ferment. glucose	ONPG	Rhamnose	Mannitol	Saccharose	Sorbitol	Inositol	Urease	P139 agar
CCM 1307	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
CCM 1318	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
43.73	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
84.74	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
44.73	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
36.73	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
91.80	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
166.74	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
88.68	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
82.68	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
81.76	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
A 141.72	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
130.73	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
72.78	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
63.80	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
122.77	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
20.78	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
67.79	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
46.79	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
39.77	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
31.79	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
83.74	C	-	+	-	+	-	-	+	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-
78.84	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
78.84 B6	B	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
87.28	C	-	+	-	+	-	-	+	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-
Sens de la réaction			-	+	-	+	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
% de souches d'Aeromonas s. répondant au test			100 %	100 %	100 %	93,6	100 %	100 %	100 %	100 %	100 %	100 %	100 %	84,9	100 %	80,6	100 %	100 %	93,6	100 %	100 %	100 %	100 %

Overall reaction

% Aeromonas strains regarding to the test

Table 1. Detection and Identification of Aeromonas salmonicida Strains

B = bacillus
C = coccus
~ = uncharacteristic reaction

	Merthiolate mg/l 0.1% Hersept(4)	Povidone-Iodine g/l 8.5% Betadine	Chlorhexidine mg/l 3% Nibitane	Quat. ammonium cpds ml/ml AMMO 4 B	Cetrimide mg/l 30% Cetavlon	Flumequine mg/l 50% Flumix	Nitrofurantoin mg/l Furadoine	Chloramphenicol mg/l Mycetine	Co-Trimoxazole mg/l Bactrim 120 mg/5 ml	Ampicillin mg/l Totapen I.V.
n°1 CCH 1307	0,5	2,12	500	1/6666	28,5	0,2	1,33	20	20	0,83
n°2 CCH 1318	0,5	2,12	500	1/6666	28,5	0,2	1,33	20	20	0,41
n°3 49.73	0,37	2,12	50	1/20000	28,5	0,1	12,5	7,5	20	<0,83
n°4 84.74	0,37	2,12	100	1/10000	28,5	0,1	12,5	> 500	20	<0,83
n°5 44.73	0,5	2,12	76,9	1/10000	28,5	0,1	12,5	7,5	5	0,33
n°6 36.73	0,75	2,12	83	<1/30000	5	0,04	8,33	500	20	<0,83
n°7 91.80	0,75	2,12	50	1/20000	28,5	0,2	12,5	> 500	20	0,31
n°8 164.74	0,75	2,12	500	1/10000	28,5	0,1	12,5	5	5	0,41
n°9 88.68	0,5	2,12	500	1/3000	40	0,1	12,5	7,5	20	0,33
n°10 62.68	0,5	2,12	100	1/10000	28,5	0,04	33	> 500	20	0,5
n°11 81.76	0,5	2,12	50	1/35000	10	0,1	12,5	500	20	0,31
n°12 A141.73	0,75	2,12	500	1/6666	28,5	0,2	12,5	> 500	20	0,83
n°13 130.73	0,5	2,12	500	1/6666	28,5	0,2	12,5	20	20	0,62
n°14 72.78	0,5	1,41	500	1/10000	28,5	0,2	12,5	20	5	0,62
n°15 65.80	0,5	1,41	500	1/6666	28,5	0,2	12,5	20	20	0,62
n°16 188.77	0,5	1,41	500	1/3000	40	0,2	8,33	500	20	0,62
n°17 80.78	0,5	2,12	500	1/3000	28,5	0,2	6,25	20	20	0,83
n°18 67.79	0,37	1,41	500	1/6666	28,5	0,1	8,33	20	20	0,83
n°19 46.79	0,5	1,41	500	1/3000	40	0,2	1,33	> 500	20	0,83
n°20 59.77	0,5	1,41	500	1/6666	28,5	0,4	8,33	20	20	1,25
n°21 31.79	0,37	1,41	50	<1/30000	5	0,04	6,25	3,75	5	0,31
n°22 99.81	0,5	1,41	100	1/6666	28,5	0,1	8,33	> 500	20	0,41
n°23 99.2484	0,5	1,41	100	1/6666	28,5	0,1	8,33	> 500	20	0,41
[illegible]	0,75	2,12	500	1/3000	40	0,4	25	> 500	20	1,25

Table 2. Determination of Minimum Inhibitory Concentrations of the Antibiotics and Antiseptics

* Effective dose producing 100% inhibition

for the most active antibiotic and 500 mg/l for the least). When the antiseptics were listed in decreasing order of effectiveness, merthiolate proved the most effective, followed by the quaternary ammonium compounds, chlorhexidine and povidone-iodine, in that order.

Discussion

A. Interpretation of Identification Reactions.

Except for strains 05.74 and GF 28, the results demonstrated the homogeneous nature of the Aeromonas salmonicida group as well as the validity of the tests employed. Maximum agreement was observed in 19 out of the 23 reactions. Our results differed little from those of Popoff, 1971 (1) in the demonstration of arginine dihydrolase, nitratase and B galactosidase levels and in saccharose use. Unlike Popoff, however, we obtained negative results with the lysine decarboxylaste test. Our findings were confirmed in Bergey's Manual, 8th edition (6).

B. Effectiveness of the Antibacterial Agents Tested

Flumequine proved completely active in doses 1,000 times lower than those considered by Michel et al (10). The effectiveness of amoicillin and nitrofurantoin has also given a new dimension to therapy. On the basis of a dose/effectiveness criterion, ampicillin and flumequine may be included among the group of decisive therapeutic agents.

Potentiation phenomena are also of interest since lower doses of sulfamide may be used when the compound is combined with trimethoprim (1,5,7,9). As part of our initial assumptions, cotrimoxazole should prove highly effective in doses of 50 mg/kg of fish/day against all the strains tested. In actual fact, this is

not the case and higher therapeutic doses are needed *in vivo*.

In contrast, chloramphenicol may facilitate the emergence of resistance even when administered in doses ten times the normal dose (cf Table 2). It is impossible to treat a furunculosis epidemic in 43.5% of the cases with the courses of treatment recommended for this antibiotic (1,5,7,8). When the dose is increased tenfold, the failure rate is still high, hovering around the 30.5% level. It should be mentioned that the strains selected may constitute cases that are refractory to conventional treatments.

Merthiolate proved to be a good antiseptic. Doses of 0.75 ml Merseptyl/litre of water produced total *in vitro* inhibition of the 23 bacterial strains. The quaternary ammonium agents also produced good results. The effectiveness of cetrimide was comparable to that of AMMO 4 8. The 1/5,000 dilution of a 20% cetrimide solution corresponded to a dose of 40 mg/l Cetavlon.

A therapeutic trial with chlorhexidine revealed that the effective dose 100 was equal to 10 ml 5% Hibitane per litre of water. Likewise, the povidone-iodine in Betadine, in concentrations of 25 ml/litre water, proved to be an excellent antiseptic.

Conclusions

During a preliminary study, 25 bacterial strains were cultured and identified. Twenty-three were found to be *Aeromonas salmonicida* organisms.

The strains responded similarly to biochemical tests, in accordance with the findings of other investigators.

Certain conclusions may be drawn regarding the use of the antibiotics we tested. Most of the treatments we selected were effective against the furunculosis-causing agent. This was not true, however, for chloramphenicol. The irresponsible and excessive use of this product has resulted in the emergence of wide-scale resistance, illustrating, once again, that certain users lack proper information on the side effects of antibiotics.

This study should open up new therapeutic perspectives provided that the possible toxicity of these substances is determined and the dosage required in fish is established.

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* Study on Aeromonas salmonicida organisms. I. Biochemical and antigenic characteristics

** Furunculosis in trout or redmouth disease

*** Use of flumequine against furunculosis in salmonids: therapeutic trials and practical perspectives